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Research Article

# Nutraceutical Importance of Dietary Curcumin, Its Analogues in Restricting Melanoma Growth and Modulation of Multiple Targets

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## Abstract

**Objectives:** Melanoma is one the most lethal form of skin cancer with a survival rate of less than 5% and thus possesses biggest challenge for its therapy among research scientists. The Melanoma progression within a tumor is guided by multiple mechanisms, thereby hindering the development of successful therapies. Therefore, there is a urgent demand to identify potential targets against melanoma cancer cells as well as to develop potent inhibitors in order to reduce melanoma at the very onset of cancer cell growth through targeting mutidrug resistance. It is well reported that curcumin is acting as inhibitor for metastasis of melanoma cancer cells. The most potential target of melanoma cancer cells has to be identified in order to reduce the relapse as well as to overcome multidrug drug resistance.

**Methods:** In this study, the potency of curcumin analogues as well as their interaction with potential targets was identified by insilico approaches.Their efficacy has been examined through invitro assay.

**Results:** Based on both invitro and insilico studies, few curcumin analogues have shown higher inhibitory activity against melanoma cancer cells than curcumin.

**Conclusion:** The present study predicts some novel curcumin analogues that can act as potent inhibitor of melanoma cancer cells.

**Keywords:** Bioavailability, cancer cells, cancer relapse, curcumin, melanoma, multidrug resistance

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Recent advancement on melanoma treatment leads to development of wide range of therapeutic approaches. It is well reported that incidence of melanoma is age and sex dependent.[1] It usually affects women by 40 years of age however the incidence highly increases for men greater than 50 years of age. The major hurdle in the melanoma treatment is the resurgence of cancer cells. The main reason of melanoma relapse lies in the enhanced multidrug resistance of cancer cells. It is utmost important to inhibit growth of cancer cells by enhancing bioavailability of anticancer drugs.

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## Melanoma Cancer Cells

According to GLOBOCAN estimation, 324,635 number of new cases (1.7%) and 57,043 number of deaths (0.6%) of melanoma cancer were reported in 2020. It was reported as the 19th most common cancer in GLOBOCAN estimate of 2008. Melanoma cancer is arises through malignant transformation of melanocytes.[2, 3]

Melanoma cells show no incidence of cancer stem cells (CSCs) instead its progression within a tumor microenvironment is mediated by clonal heterogeneity within a given population of tumor cells. Their growth mainly depends on factors that constitute the niche inside the tumor. Cancer cell niche is a part of tumor microenvironment that composed of extracellular matrix, immune cells, various growth factors, cytokines and fibroblasts.[4] The significant role of melanoma cancer cells lies in maintaining heterogeneity as well as promoting epithelial-mesenchymal transition and metastasis of cancer cells.[5] The process of epithelial-mesenchymal transition is a biological process in which epithelial cells acquire properties of mesenchymal properties during embryonic development.[5]

In melanoma, the epithelial-mesenchymal transition process enhances cell migration and invasion of cancer cells.[6, 7] Identification and targeting of potential targets of melanoma cancer cells are significantly important approaches for improving melanoma treatment.[8]

## Curcumin

Although there are wide ranges of chemical compounds that perform inhibitory activity on the overexpression of critically important proteins in melanoma, the modulatory activity of curcumin is well reported. Curcumin (Fig. 3a) is an polyphenolic compound which is extracted from *Curcuma longa*. It exhibits antioxidant, anti-inflammatory, antiproliferative, anti-angiogenic, pro-apoptotic and immunomodulatory properties against several cancers including melanoma.[9] In order to prevent drug resistance in melanoma cancer cells, the modulation of multi-targeted curcumin has great importance in reducing melanoma cancer cells growth.[10] Due to its low absorption in intestine and rapid metabolism in liver, it runs with less retention time in the peripheral target tissues/cells.

## Effects of Curcumin on Molecular Targets of Melanoma Cancer Cells

Curcumin exhibits inhibitory activity on multiple targets including estrogen receptor (ER), Insulin-like growth factor receptor (IGF-1R), motor protein of human kinesin family, P-glycoprotein, Matrix metalloproteinase-10, mammalian target of rapamycin (mTOR), Bcl-XL protein, extracellular signal-regulated kinase2 (ERK2), p21-activated kinases (PAK1) and Epidermal Growth Factor Receptor (EGFR). Mutation rates in melanoma are often very high as compared to other forms of aggressive tumors thereby making it difficult to screen and to predict therapeutic efficacy of drug responses. Furthermore, multiple signaling pathways get implicated as a result of heterogeneous mutations.[11] So far in most of the cases melanoma has been linked to oncogenic mutations in BRAF and NRAS oncogenes along with over-activation of MAPK/ERK and PI3K/Akt signaling pathways.[12]

Estrogen exerts their biological effects through its receptors, estrogen receptor (ER) including α and β. ER are transcription factors belonging to nuclear receptor subfamily.[13] Several studies suggest expression of ERβ is predominantly associated with melanoma cancer tumor microenvironment. ERβ is ubiquitously expressed by sebaceous glands, hair follicles, epidermis, eccrine glands, vessels and fibroblasts.[14-18] In vitro study on BLM (BRAF-wild type, NRAS-mutant) melanoma cells suggests that ERβ, agonists [17β-estradiol, diarylpropionitrile (DPN), KB1] significantly inhibits cell proliferation accompanied by an altered expression of the proteins involved in the G1/S transition of the cell cycle (decreased levels of cyclin D1 and cyclin D3, and increased expression of p27, a CDK inhibitor). Similar observations were previously reported in different types of cancers expressing this receptor.[19] Therefore, it can be anticipated that natural plant products that preferentially binds to the ERβ ligands might be related to novel therapeutic strategy in modulating specific oncogenic mutation in melanoma. The anti-proliferating activity of curcumin depends on estrogen presence.

The insulin-like growth factor receptor (IGF) belongs to tyrosine kinase family and act as cell proliferation mediator. Upregulation of IGF-1R has been reported in melanoma acquiring resistance to inhibitors such as SCH772984 (Erk1/2 inhibitor), vemurafenib (BRAF inhibitor) and trametinib (MEK inhibitor).[20] IGF has shown key role in promoting tumorigenesis and cell proliferation. The significance of IGF-1R lies in altering drug resistance activity of cancer cells.[21] Curcumin exerts significant anti-cancerous activity by inhibiting IGF-1 receptor.[22, 23]

Molecular motor proteins of kinesin family exhibit ATP-dependent activity. In melanoma cells, the overexpression of these motor proteins stimulates resistance to anticancer drugs.[24] High expression of P-glycoproteins reduces bioavailability of anti-cancer drugs via providing protection for cancer cells.[25, 26]

Multiple forms of matrixmetalloproteinase (MMPs) activate several proteolytic enzymes, release active factors to remodel the extracellular matrix, and also subdue cell surface receptors thereby mediating melanoma cross communication within the tumor microenvironment. In vitro study suggests inhibiting the active sites in MMPs could also be of importance in future therapeutics.[27, 28] According studies of Banerji et al., curcumin has significant inhibitory effect

on MMP-2 on metastatic melanoma cells B16F10.[29]

It is well reported that the aberrant expression of Mammalian target of rapamycin (mTOR) activity regulates self-renewal and metastasis in melanoma cancer cells.[30] Based on recent studies, curcumin reduces drug resistance in melanoma cancer cells by regulating Bcl-XL.[31]

It has been reported that activation of the ERK/MAP-kinase (MAPK)-pathway, is a major contributor to melanoma pathogenesis and progression.[32] The signaling of ERK/MAP-kinase (MAPK) pathway is further amplified by phosphorylation of ERK by p-AKT which remains hyperactive in most of the cancers including melanoma.[12] Aberrant PI3K-AKT signaling in melanoma can lead to up-regulation of SLUG expression, which appears to be driven by osteonectin (also known as SPARC), a secreted extracellular matrix-associated factor that promotes the epithelial-to-mesenchymal transition.[33] Curcumin inhibits proliferation, invasion and angiogenesis by suppressing ERK protein.[34]

Receptor like p21 activated kinases (PAKs) is critically involved in tumorigenesis and cancer progression. PAKs are serine/threonine-specific protein kinases that involve in cell migration and proliferation.[35] The overexpression of PAK1 leads to apoptosis inhibition, cancer invasion and metastasis.[36] Curcumin inhibits PAK activation through inducing proliferation and invasion.[37]

EGFR signaling is found to be over activated in more than 80% melanoma. Owing to BRAF inhibition in melanoma, EGFR signaling enables feedback re-activation of the MAPK pathway and activation of the PI3K–AKT pathway. Thus, EGFR signaling plays a crucial role in development of resistance to MAPK pathway inhibitors in melanoma.[38] In melanoma therapy, curcumin was found to be effective inhibitor of EFFR on B16F10 melanoma cancer cell line.[9]

This present work finds the effect of curcumin and its derivatives on reducing multidrug resistance and enhancing bioavailability of anticancer drugs in melanoma has been determined.

## Materials and Methods

### Chemical Synthesis

#### Synthesis of Curcumin (1)

Curcumin was synthesized according to the Patent WO 2007/110168A1.To the stirred solution of vanillin (15.2g,100mmol), acetylacetone (5.2g, 52mmol), boric acid (6g,97mmol), m-xylene (3.3g), dimethyl formamide (18ml),and N-butylamine (1.0ml,10mmol) at 80-85 °C under reduced pressure for 2h,n-butyl amine (0.5ml, 5mmol) in dimethylformamide (10 ml) was added and the mixture refluxed for another 2h.After completion of reaction as indicated by TLC, the reaction mixture was diluted with acetic acid: water ( 45 ml ; 92:8,v/v) while stirring for 30 min. The reaction mixture was poured in acetic acid: water (10:90) and stirred for 2h at 80°C. Reaction mixture was filtered and residue washed with water, and crude product recrystallized from methanol, and confirmed by1H NMR spectroscopy.$^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.61 (d, J = 15.8 Hz, 2H), 7.19 – 7.03 (m, 4H), 6.96 (d, J = 8.2 Hz, 2H), 6.50 (d, J = 15.8 Hz, 2H), 5.87 (d, J = 34.5 Hz, 2H), 3.97 (s, 6H).

#### Synthesis of Mono and Di Glucosyl Curcumin

A modified procedure of Koenigs–Knorr was followed for the synthesis of glucosylated curcuminoids. The reaction was initiated by adding aqueous KOH (100ml;10.8 mmol) to dichloromethane (100ml) containing curcumin (2.4g, 6.5mmol), followed by the addition of 2,3,4,6-tetra-O-acetyl-D-glucopyranosyl bromide (6g, 13mmol) and benzyl tributyl ammonium chloride (2g, 6.4mmol). The reaction mixture was stirred for 5h at rt under nitrogen atmosphere. On completion of the reaction, the products, curcumin-di-glucoside tetra acetate (1a) and curcumin-mono-glucosidetetra acetate (1b) were isolated .These compounds were deacetylated by adding 2% sodiummethoxide in dry methanol and acidic dovex resin. The two products formed were isolated and purified through column chromatographyusing silica gel (100–200 mesh). The structures of the compounds obtained i.e. curcumin-di-glucoside (2a) and curcumin-mono-glucoside (2b) were confirmed by NMR, LC–MS studies as given in Figure 1.

### Computational Analysis

#### Target Preparation

Three-dimensional structures of ten target proteins were downloaded from Protein Databank (PDB). The downloaded protein structures were prepared on Protein preparation module of Schrodinger.

#### Ligand Preparation

The two-dimensional structures of curcumin analogues were drawn on ChemDraw. Using LigPrep module, possible conformers were generated with OPLS_2005 force field. Curcumin analogues were prepared to perform docking and simulation study.

#### Docking and Simulation Study

The docking of simulation study of downloaded proteins and ligands was carried out using Schrodinger's Glide (Glide-based Ligand Docking with Energetics) module. The receptor grids were generated for target proteins. The optimized conformers of protein and ligand complex were predicted with extra precision docking.

### Cell Line Analysis

MDA-MB-435s, melanoma cell line was procured from NCCS, Pune, India. The celllines were found to be free from

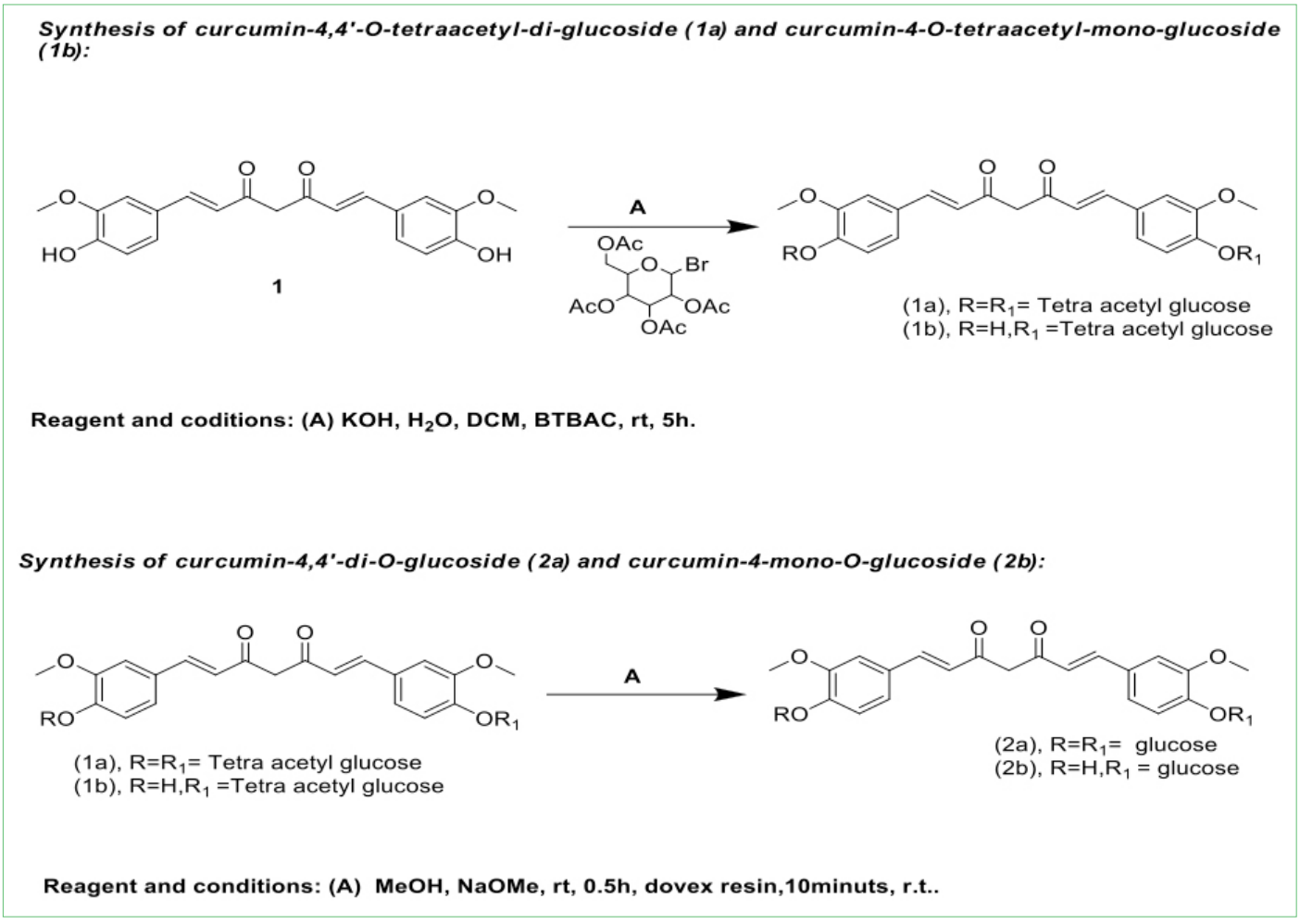


**Figure 1.** Synthesis of curcumin analogues.

microbial and cross contaminations. It was maintained in Leibovitz's L-15 medium alongwith 10% FBS and antibiotics (penicillin and streptomycin; 100 µg/ml each). Cells viability was estimated by Trypan blue exclusion test and then seeded onto plate of 96 wells in 100 µL of Leibovitz's L-15 complete culture media. The cells were then kept to settle in a incubator (5% $CO_2$ at 37°C) for 12h. After overnight incubation, various concentrations ($10^{-7}$ to $10^{-4}$ M) of curcumin, curcumin monoglucoside, curcumin diglucoside, prepared by serial dilutions with culture medium, were added on plates and incubated for another 24h. Using MTT assay, the viability of cells was determined which works on the reducing ability of viable cells into formazan crystals (blue) from soluble tetrazolium salt (yellow). MTT dye (5mg/ml, 10 µl/100 µl media), composed of phosphate buffered saline (PBS), was equally added to wells after 24h of treatment. Plates were incubated for the period of 4h in incubator. Then the media was discarded and purple-colour precipitates of formazan was dissolved in 100 µL of dimethyl sulfoxide (DMSO) followed by incubation for 10 min with gentle shaking. At 570 nm, optical density was determined on a synergy H1 hybrid microplate reader. By using Graphpad Prism5 software, the semi logarithmic dose–response plots were constructed to determine the $IC_{50}$ values of compounds.

## Results and Discussions

### NMR Spectra of Synthetic Compounds

**Compound (1)** $^1$H NMR (400 MHz, $CDCl_3$) δ 7.61 (d, J = 15.8 Hz, 2H), 7.19 – 7.03 (m, 4H), 6.96 (d, J = 8.2 Hz, 2H), 6.50 (d, J = 15.8 Hz, 2H), 5.87 (d, J = 34.5 Hz, 2H), 3.97 (s, 6H).

**Compound (2)** Yellow colored solid, ESI-MS (m/z): 531 (M+1).$^1$H NMR (800 MHz, MeOD) δ 7.59 (dd, J = 15.7, 9.3 Hz, 2H), 7.28 (s, 1H), 7.22 (s, 1H), 7.12 (d, J = 8.0 Hz, 2H), 7.1 (s, 1H), 6.83 (d, J = 8.1 Hz, 1H), 6.70 (d, J = 15.8 Hz, 1H), 6.65 (d, J = 15.7 Hz, 1H), 4.99 (d, J = 7.6 Hz, 2H), 4.65 (s, 1H), 3.92 (d, J = 3.0 Hz, 6H), 3.90 (dd, J = 7.6, 5.5 Hz, 1H), 3.72 (dd, J = 12.2, 5.6 Hz, 1H), 3.56 – 3.52 (m, 1H), 3.50 (dd, J = 15.6, 6.6 Hz, 1H), 3.48 – 3.45 (m, 1H), 3.42 (dd, J = 11.8, 6.7 Hz, 1H), 3.33 – 3.31 (m, 2H). $^{13}$C NMR (201 MHz, MeOD) δ 182.51, 182.49,

149.58, 149.15, 148.45, 148.01, 141.14, 139.73, 129.94, 127.09, 122.84, 122.39, 122.07, 120.88, 115.95, 115.17, 110.92, 110.30, 100.77, 76.88, 76.43, 73.40, 69.86, 61.06, 55.32, 55.02.

**Compound (3)** ESI-MS (m/z): 715 $(M+Na)^{+}$.$^{1}$H NMR (400 MHz, DMSO) δ 7.59 (d, J = 15.8 Hz, 2H), 7.38 (s, 2H), 7.25 (d, J = 8.4 Hz, 2H), 7.13 (d, J = 8.4 Hz, 2H), 6.87 (d, J = 15.9 Hz, 2H), 6.13 (s, 1H), 5.32 (s, 2H), 5.14 (s, 2H), 5.07 (d, J = 5.0 Hz, 2H), 5.00 (d, J = 6.3 Hz, 2H), 4.59 (t, J = 5.3 Hz, 2H), 4.1 ( br, s,1H) 3.84 (s, 6H), 3.28 (s, 8H), 3.17 (d, J = 4.8 Hz, 3H).

## Docking Study on Multiple Targets of Cancer Cells

To analyse the interaction between potential molecular targets of melanoma cancer cells and curcumin analogues, the docking and simulation study has been performed. Due to potential therapeutic role of molecular targets in the progression of melanoma, ten potential targets have been considered as shown in Table 1. This table shows the docking scores of curcumin and curcumin monoglucoside on selected molecular targets of melanoma.

In this docking results, the binding affinity of curcumin and monoglucoside curcumin have been analyzed. On all targets of melanoma cancer cells including Estrogen receptor (PDB ID: 1UOM), Insulin-like Growth Factor receptor (PDB ID: 2ZM3), Human Kinesin protein (PDB ID: 3B6V), P-glycoprotein (PDB ID: 3G60), Matrix metalloproteinase-10 (PDB ID: 3V96), mTOR kinase (PDB ID: 4JSV), Bcl-XL (PDB ID: 4QNQ), ERK2 (PDB ID: 5AX3), PAK1 (PDB ID: 5DFP) and EGFR Kinase (PDB ID: 5HIB). The docking score of monoglucoside curcumin was found to be lower than curcumin on all selected targets of melanoma cancer cells. This suggests higher inhibitory activity of monoglucoside curcumin against potential molecular targets of melanoma. Based on our previous research work, the inhibitory activity of monoglucoside curcumin was found to be more effective than diglucosidecurcumin and curcumin.[39] Figure 2 represents the interaction between target P-glycoprotein and

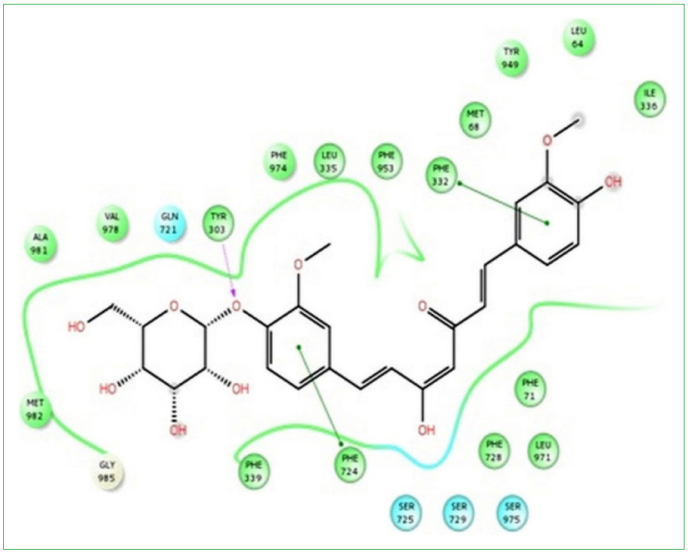


**Figure 2.** Interaction of curcumin-monoglucoside and P-glycoprotein.

**Table 1.** Docking results of curcumin analogues on multiple molecular targets of melanoma

| S.N | Molecular Targets | PDB IDs | Ligands | GScore | Hbond |
|---|---|---|---|---|---|
| 1. | Estrogen receptor | 1UOM | Monoglucosidecurcumin | -10.3 | -3.9 |
| | | | Curcumin | -9.3 | -1.0 |
| 2. | Insulin-like Growth Factor receptor | 2ZM3 | Monoglucosidecurcumin | -9.7 | -4.0 |
| | | | Curcumin | -6.1 | -0.5 |
| 3. | Human Kinesin protein | 3B6V | Monoglucosidecurcumin | -10.9 | -3.8 |
| | | | Curcumin | -8.6 | -2.2 |
| 4. | P-glycoprotein | 3G60 | Monoglucosidecurcumin | -11.7 | -3.0 |
| | | | Curcumin | -9.8 | -3.9 |
| 5. | Matrix metalloproteinase-10 | 3V96 | Monoglucosidecurcumin | -9.8 | -9.8 |
| | | | Curcumin | -7.2 | -1.7 |
| 6. | mTOR kinase | 4JSV | Monoglucosidecurcumin | -6.1 | -3.4 |
| | | | Curcumin | -3.1 | -1.3 |
| 7. | Bcl-XL | 4QNQ | Monoglucosidecurcumin | -10.2 | -3.9 |
| | | | Curcumin | -9.4 | -2.6 |
| 8. | ERK2 | 5AX3 | Monoglucosidecurcumin | -5.7 | -2.9 |
| | | | Curcumin | -4.5 | -1.9 |
| 9. | PAK1 | 5DFP | Monoglucosidecurcumin | -7.6 | -3.4 |
| | | | Curcumin | -6.1 | -2.3 |
| 10. | EGFR Kinase | 5HIB | Monoglucosidecurcumin | -10.7 | -4.7 |
| | | | Curcumin | -8.6 | -1.6 |

curcumin monoglucoside. The interacting residues such as TYR303, PHE332, PHE953, PHE339 and PHE 724 are showing hydrophobic nature of binding site of target protein.

## Melanoma Cell Line Assays

Previous studies have shown anti-cancer and anti-tumoral effects of curcumin on B16-R and A375 melanoma cancer cell lines.[40, 41] $IC_{50}$ values against MDA-MB-435 cell line were determined in the presence of curcumin and its derivatives. As shown in Figure 3 (a-c), the results of MTT assay suggest that after 24h of incubation, curcumin (Fig. 3a) curcumin- monoglucoside (Fig. 3b) and curcumin-diglucoside (Fig. 23) showed $IC_{50}$ values of 12.0 x $10^{-4}$ M, 6.5 x $10^{-5}$ M and 1.0 x 10-4 M respectively. The values of $IC_{50}$ indicate that curcumin derivatives act as stronger cytotoxic agents than the natural curcumin when tested against MDA-MB-435s cell line.

## Conclusion

In the present work, both *in-silico* and *in-vitro* approaches have been used to enhance bioavailability as well as to increase anticancer effects of curcumin and its analogues, i.e. mono and diglucosides. Based on findings of previous in-silico study, it has been found that curcumin monoglucoside has highest inhibitory effect on melanoma cells in comparison to curcumin and its diglucoside. The findings of present *in-vitro* work validates the higher bioavailability and inhibitory activity of curcumin-monoglucoside against growth of melanoma cancer cells. This can be further exploited to reduce proliferative effects on melanoma cells via modulating multidrug resistance therapy. Probably both factors i.e. inhibition of efflux through binding with P-gp and improved cellular uptake due to presence of glucose moiety are effective in the present case.

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**Disclosures**

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**Conflict of Interest:** None declared.

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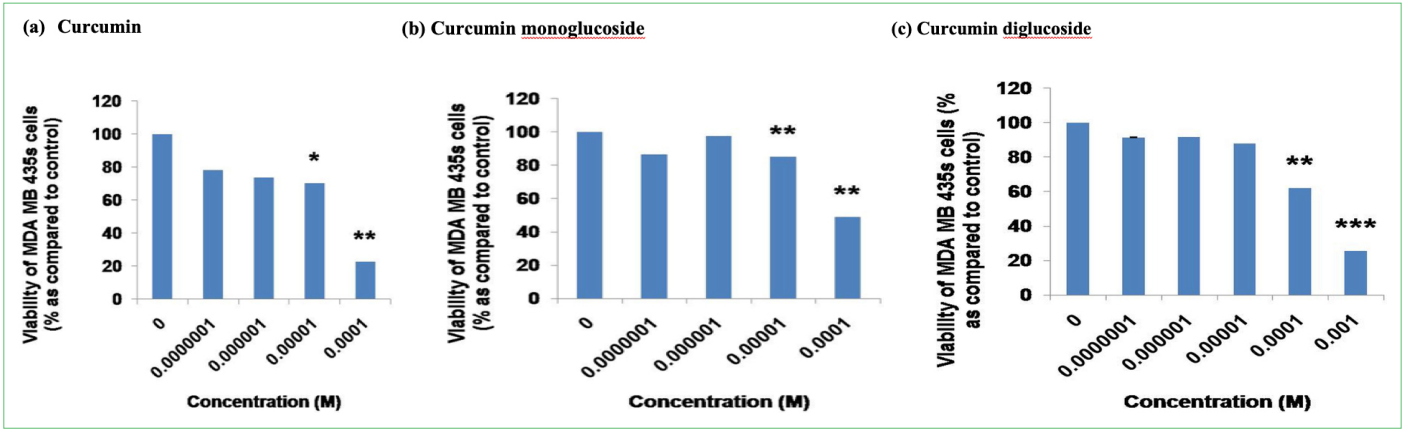


**Figure 3.** The histogram represents the cytotoxcity of curcumin **(a)**, curcuminmonoglucoside **(b)** and curcumindiglucoside **(c)** against MDA-MB-435S cell viability after 24 hours of treatment. The data represents mean±SD (n=3) percent of viable cells considering the untreated control as 100%.

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